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Citation for published version:

Gossner, AG, Venturina, VM, Peers, A, Watkins, CA & Hopkins, J 2012, 'Expression of sheep interleukin 23 (IL23A, alpha subunit p19) in two distinct gastrointestinal diseases', *Veterinary Immunology and Immunopathology*, vol. 150, no. 1-2, pp. 118-122. <https://doi.org/10.1016/j.vetimm.2012.08.004>

Digital Object Identifier (DOI):

[10.1016/j.vetimm.2012.08.004](https://doi.org/10.1016/j.vetimm.2012.08.004)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Early version, also known as pre-print

Published In:

Veterinary Immunology and Immunopathology

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Expression of sheep interleukin 23 (IL23A, alpha subunit p19) in two distinct
gastrointestinal diseases

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Abstract

This paper reports the sequence of sheep interleukin 23A (p19), and shows that it shares 98% identity with bovine IL23A, 85% with human and 76% with mouse IL23A. It also reports the existence of two allelic variants that differ largely within the region encoding the amino terminal polypeptide signal sequence. An optimized RT-qPCR assay was used to quantify IL23A transcripts in sheep infected with two common gastrointestinal pathogens, the intracellular bacterium *Mycobacterium avium* subspecies *paratuberculosis* and the parasitic nematode *Teladorsagia circumcincta*. No differential expression of IL23A was detected in the mesenteric lymph node of sheep with the different pathogenic forms of paratuberculosis, however significantly high levels of IL23A were detected in the ileal mucosa of the paucibacillary form in comparison with the asymptomatic or multibacillary forms. Similarly, significantly high levels were present in the gastric lymph node draining *Teladorsagia circumcincta*-infected abomasum in susceptible sheep. High levels of IL23A seem to be associated with lymphocytic infiltration and inflammation in both diseases but not with the macrophage infiltrate of multibacillary paratuberculosis.

Keywords: sheep; cytokines; IL23A; inflammation; mucosal immunity.

Introduction

Control of different pathogens is dependent on the ability of the immune system to differentially coordinate the generation of optimal antigen-specific immune mechanisms (Kastelein et al., 2007). This is illustrated by two common gastrointestinal pathogens of sheep; the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (Map) and the nematode parasite *Teladorsagia circumcincta*. Map infects macrophages in the lower ileum and causes paratuberculosis or Johne's disease (Clarke, 1997) and the abomasal parasite, *T. circumcincta* is the most common cause of parasitic gastroenteritis of sheep in cool temperate regions (Urquhart et al., 1987). Infection of sheep with Map can result in one of two pathologies; paucibacillary (tuberculoid) disease is linked to control of mycobacterial replication by a Th1 response while a highly polarized Th2 response and high antibody levels results in multibacillary (lepromatous) disease (Burrells et al., 1999; Smeed et al., 2007) associated with uncontrolled bacterial growth. In contrast, elimination of *T. circumcincta* after acute infection or resistance after continual infection is mediated by Th2 cytokines and IgA antibody (Craig et al., 2007; Beraldi et al., 2008) and susceptibility to continual infection is associated with persistent inflammation (Gossner et al., 2012). T cell differentiation and polarization within the gastrointestinal tract is highly regulated to optimize protective immunity and minimize immune pathology (Ahern et al., 2008). A major component of this regulation involves macrophages and dendritic cells (DC) within the mucosal lamina propria (Hunter, 2005; Kastelein et al., 2007; Coombes and Powrie, 2008), through the production of IL-12 and IL-23 and the activation of Th1 cells via the CD40-CD154 (CD40L) pathway (Uhlig et al., 2006). IL-12 and IL-23 are closely related dimeric cytokines, each being composed of one unique molecule, IL-12A (IL-12p35) and IL23A (p19), associated with the common IL-12B (IL-12p40) (Langrish et al., 2004). The major functional difference between these two cytokines involves the activation of Th17 cells by IL-23 (Uhlig et al., 2006; Kastelein et al., 2007), which have major inflammatory functions (Korn et al., 2009).

Map has been implicated in the pathogenesis of a variety of inflammatory bowel diseases (Chacon et al., 2004; Davis and Madsen-Bouterse, 2012) and IL-23 has been shown to be a key regulator of inflammation at mucosal surfaces, where dysregulation is linked to these diseases (Ahern et al., 2010; Geremia et al., 2011). IL-12 is not differentially expressed between the pauci and multibacillary forms in sheep paratuberculosis (Smeed et al., 2007) or between *T. circumcincta* infected susceptible and resistant sheep (Gossner et al., 2012) implying a possible role for IL-23 in the discrimination of the pathologies. In this study we report the nucleotide and predicted protein sequences of two alleles of sheep IL23A and

compare these sequences to those in related species. An absolute quantitative RT-qPCR was developed and was used to quantify differential expression of IL23A transcripts in the different pathological forms of sheep paratuberculosis and in resistant and susceptible lambs continually infected with *T. circumcincta*.

Materials and methods

Animals and tissues

Terminal ileum and mesenteric lymph nodes (MLN) were from Map infected animals that presented with clinical paratuberculosis; all were female of a variety of breeds and ages (Table S1). Sheep were euthanized and diagnosis of pathology was by histopathology, Ziehl-Neelsen (ZN) staining and IS900 real-time quantitative PCR as described previously (Smeed et al., 2007; Nalubamba et al., 2008; Smeed et al., 2009). Sheep of similar ages with no signs of clinical disease and negative for IS900 DNA were considered uninfected controls (n = 6 for each group). Gastric lymph nodes (GLN) were from *T. circumcincta* – infected female Blackface lambs that had been described previously (Beraldi et al., 2008). Ten lambs were sham-infected controls and 45 lambs were infected with ~2300 infective L3 larvae three times a week for 12 weeks and sacrificed after 13 weeks. At *post mortem* ten infected lambs had no detectable adult worms in the total abomasal contents (resistant group) and a mean faecal egg count (FEC) level of 8 per gram faeces (0 – 80).

The ten lambs in the susceptible group had a 93 mean of 5960 adults worms (4200 – 11300) and a mean FEC of 330 (75 – 950). Tissues were removed from animals immediately post-mortem and 5 mm blocks stored in RNAlater (Ambion, Huntingdon, UK) at –80°C. Animal experiments were approved by University of Edinburgh Ethical Review Committee and conducted under an Animals (Scientific Procedures) Act 1986 Project Licence.

Cloning and sequencing of full length IL23A

Complementary DNA from sheep lymph node was synthesized with Superscript III using oligo-dT (Invitrogen, Paisley, UK) and reverse transcribed using gene specific primers with Transcriptor Reverse Transcriptase (Roche Diagnostics Ltd., Lewes, UK). RT-PCR was performed using primers based on the bovine IL23A sequence (Chen et al., 2009)(GenBank acc # NM_001205688.1) to amplify a partial sheep IL23A cDNA sequence; forward 5'-CAGCTCTCACAGCAACTCTGC-3' and reverse 5'-GGTCAACATCGTCAGTCAGTCAG-3'. The amplicons were cloned into pGEM-T Easy (Promega, Southampton, UK) and sequenced on both strands using T7 and SP6 primers in separate reactions with BigDye®

Terminator v3.1 (Applied Biosystems, Warrington, UK). Full length sheep IL23A sequence was obtained using the 5'/3' RACE kit (2nd Generation, Roche); exploiting three gene specific primers designed from the partial sequence. The amplicons were cloned and sequenced as previously described; the sequences were compiled using CLC Main Workbench v6.6.2 (www.clcbio.com).

Quantitative RT-PCR analysis

Primers were F:ACCTGTGAGCCAATGAGTTC; R: GGTCAACATCGTCAGTCAGTC and were selected using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The forward and reverse primers represent 784 – 803 and 856 – 876 of ovine IL23A (Genbank acc # NM_001185122.2) respectively and quantified the expression of both IL23A alleles. RT-qPCR for IL23A in *T. circumcincta* tissues was performed exactly as described previously (Gossner et al., 2012). For the paratuberculosis 119 tissues, cDNA from sheep lymph node or terminal ileum was synthesized using 1 µg of total RNA with the Superscript VILO kit as described by the manufacturer (Invitrogen, Paisley, UK). All PCR reactions were performed in 15 µl final volume containing 2.5 µl template cDNA diluted 1/40) or linearized plasmid DNA, 7.5 µl EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Invitrogen) and primers at 0.4 µmole. The amplification profile was, 2 min at 95°C, 40 cycles of 10 s at 95°C and 50 s at 63°C, followed by dissociation curve analysis to confirm a single gene product. Copy number levels were quantified three times in separate RT-qPCR runs, each time using cDNA from a different RT reaction and within a run, each sample was assayed in triplicate. Reference genes (GAPDH and YWHAZ for ileum and GAPDH and HPRT for lymph node) were selected using GeNorm v3.4 (Vandesompele et al., 2002) and the NormFinder Microsoft Excel applet (Andersen et al., 2004). Normalized copy numbers were obtained using the normalization factor determined by GeNorm. The expression levels were normalized by dividing the copy number derived from the standard curve by the calculated normalization factor for each individual sample. Statistical differences between groups were determined by one-way ANOVA with Tukey-Kramer's post-hoc test for multiple pairwise comparison analysis.

Results and discussion

Characterization of sheep IL23A

A 579-nucleotide open reading frame (NM_001185122.2) (sheep allele 1) was predicted (ORF Finder) to encode a 192 amino acid polypeptide with a calculated M_r 21,250 and isoelectric point equal to 6.14 (GenPept acc # NP_001172051.1). A putative signal peptide of 21 amino acids was predicted using SignalP 3.0 Server (Emanuelsson et al., 2007), with cleavage between G and R, the same position as in other mammalian IL23A sequences. Quantitative sequence alignment was performed using Emboss::needle (<http://www.ebi.ac.uk/Tools/emboss/align/>) and showed that the sheep IL23A sequences are 98% and 97% identical to the cattle coding region nucleotide and amino acid (Genbank acc # NP_001192617) sequences respectively. There are six differences between the sheep and cattle amino acid sequences; semi-conservative substitutions at positions 6 (V/A), 33 (A/T), 119 (F/L), 147 (A/T) and conservative substitutions at positions 66 (E/K) and 121 (D/N). A second sheep IL23A sequence (sheep allele 2) was submitted recently (Genbank acc # JN935767.1) with an open reading frame of 573 nucleotides encoding a predicted 190 amino acid polypeptide (GenPept acc # AEV89921.1). This is similar in size to the annotated IL23A gene in Sheep Genome v3.0 (OAR3) (<http://www.livestockgenomics.csiro.au/sheep/oar3.0.php>). The two alleles differ by 6 nucleotides (ATGCTG) at position 22 to 27 of the coding region, leading to a deletion of a methionine and a leucine at positions 8 and 9 of the signal peptide (Fig. 1). In addition there are two non-synonymous changes at positions 11 (A/G) and 17 (T/C) of the coding region leading to N – S and V - A substitutions at positions 4 and 6 respectively. The two sheep IL23A amino acid sequences have 6 or 7 leucines between positions 8 – 16 of the signal peptide; other non-rodent IL23A sequences have between 5 (human) and 8 (dog) leucine residues at the same region. The functional consequences of the two sheep IL23A alleles are currently unknown.

Sheep IL23A allele 1 shares 98% and 97% identity with *Bos taurus* IL23A nucleotide (XM_588269.4) and amino acid (NP_001192617) sequences respectively, and more distantly 85% (80%) with *Homo sapiens* and 76% (64%) with *Mus musculus*. Despite the fact that both IL-23A and IL-12A interact with IL-12B to form functional heterodimers, the phylogenetic relationship between the two (13.8% identity) is only marginally closer than IL23A and IL12B (12.1%) and is more distant than IL23A and GCSF (22%), IL27 (24%) and IL6 (19.2%). Because of the 6 nucleotide deletion and two non-synonymous substitutions, at positions 566 (T - C) and 572 (C - T), sheep allele 2 shares 99% (96%) identity with sheep

allele 1 and only 97% (93%) identity with bovine IL23A. Blast analysis against Sheep Genome v3.0 (<http://www.livestockgenomics.csiro.au/blast/>) shows that sheep IL23A is encoded by four exons on Oar3 negative strand at chromosomal positions 162939104 – 162937330.

IL23A expression in two gastrointestinal diseases

The Map-infected animals were analysed in four groups; uninfected controls (IS900 negative), asymptomatic (IS900+, no pathology), paucibacillary and multibacillary (Clarke and Little, 1996; Smeed et al., 2007); *T. circumcincta*-infected animals were analysed in three groups, uninfected controls, resistant and susceptible lambs. In Map-infected sheep the levels of IL23A transcript expression in the ileal mucosa (Fig. 2A) were significantly lower than in the MLN ($P \leq 0.0001$) (Fig. 2B); although significant differences between pathological groups were only seen with the ileal mucosa. IL23A levels in the ileal mucosa of paucibacillary sheep were 1964 ± 979 transcripts per μg of total RNA, significantly higher than the infected asymptomatic (204 ± 75) and uninfected controls (228 ± 168) (both $P \leq 0.0005$), and also the multibacillary group (1035 ± 850) ($P \leq 0.05$). In *T. circumcincta*-infected animals, significant differences in IL23A transcript expression were measured in the GLN that drains the abomasum, where infected susceptible animals expressed 1319 ± 586 transcripts per μg of total RNA, significantly higher than either infected resistant (329 ± 95) and uninfected control (522 ± 297) lambs (both $P \leq 0.0005$). The levels of IL23A in abomasal mucosa were below the threshold of detection.

IL-23 is produced largely by DC and macrophages (Kastelein et al., 2007), and the high levels of IL23A in the ileal mucosa of diseased animals reflects Map-induced inflammation at this site. However, the level in the macrophage infiltrates of the multibacillary lesions are significantly lower than in paucibacillary lesions, that consist largely of T cell infiltrates (Smeed et al., 2007). A possible explanation is that the macrophages in paucibacillary lesions are largely uninfected and are associated with the induction of a type 1 T cell response that controls mycobacterial replication (Smeed et al., 2007) through the action of $\text{IFN}\gamma$. Multibacillary disease is linked to a strong type 2 response and consequently high levels of antibody, which is not able to control bacterial proliferation. The epithelioid-macrophages of the multibacillary lesions are highly infected and therefore possibly dysfunctional. The site of the immune response to Map infection is presumably the draining MLNs, which show no difference in IL23A transcript expression between the four groups. This is consistent with mice infected with *Mycobacterium t 197 tuberculosis*, where IL-23 plays little role in

mycobacterial immunity in the presence of an active IL-12 response (Chackerian et al., 2006). In contrast, IL23A is differentially expressed in the draining lymph node of *T. circumcincta* infected sheep; it is significantly increased in the susceptible lambs, which generate a persistent inflammatory response within the abomasal mucosa but fail to control nematode colonization and egg production (Gossner et al., 2012).

In this paper RT-qPCR quantified significantly high levels of IL23A transcripts in the gut mucosa in the paucibacillary form of paratuberculosis and in susceptible sheep persistently infected with *T. circumcincta*. High IL23A levels are associated with lymphoid inflammatory infiltration in these two diseases, which in paratuberculosis inhibits bacterial proliferation but in parasitic gastroenteritis fails to control nematode infection. Two alleles of sheep IL23A (p19) are characterized, which largely differ within the coding region of the signal peptide. Work is on-going to investigate the biological relevance of these two alleles especially their relationship with the different pathologies of paratuberculosis and parasitic gastroenteritis.

Acknowledgment

This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant to The Roslin Institute. VMV is a Ford Foundation (<http://www.fordfound.org/>) International Fellow. Thanks to Lisa Watson for assistance in RNA extraction from Map infected tissue.

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Figure Legends

Fig. 1. (A) 5' nucleotide sequences and (B) amino-terminal predicted peptide sequences of sheep and cattle IL23A.

Fig. 2. IL23A transcript expression, copy number per μg total RNA, in: (A) ileal mucosa, (B) MLN, of Map-infected and control sheep ($n = 6$) and (C) GLN of *T. circumcincta* infected and control lambs ($n = 10$). * $P \leq 0.05$, *** $P \leq 0.0005$ for the individual comparisons.

Fig.1

A 5' nucleotide sequences

```
Sheep1 allele 1 ATGCTGGGGAACAGAGTTGTGATGCTGCTGCTACTGCTACTGCTGCCCTGGACAGCTCAG 60
Sheep2 allele 2 ATGCTGGGGAGCAGAGCTGTG-----CTGCTACTGCTACTGCTGCCCTGGACAGCTCAG 54
OAR3           ATGCTGGGGAACAGAGTTGTG-----CTGCTACTGCTACTGCTGCCCTGGACAGCTCAG 54
Bovine        ATGCTGGGGAACAGAGCTGTGATGCTGCTGCTGCTGCTACTGCTGCCCTGGACAGCTCAG 60
               *****      *****      *****      *****      *****
```

B amino-terminal predicted peptide sequences

```
Sheep allele 1  MLGNRVVMLLLLLLLLPWTAQGRAVSEDSSP 30
Sheep allele 2  MLGSRAV--LLLLLLLPWTAQGRAVSEDSSP 28
OAR3           MLGNRVV--LLLLLLLPWTAQGRAVSEDSSP 28
Bovine         MLGNRAVMLLLLLLLLPWTAQGRAVSEDSSP 30
               ***.*.*      *****
```

alignments calculated using ClustalW2.

* nucleotides are identical in all sequences . semi-conserved substitutions

Fig.2

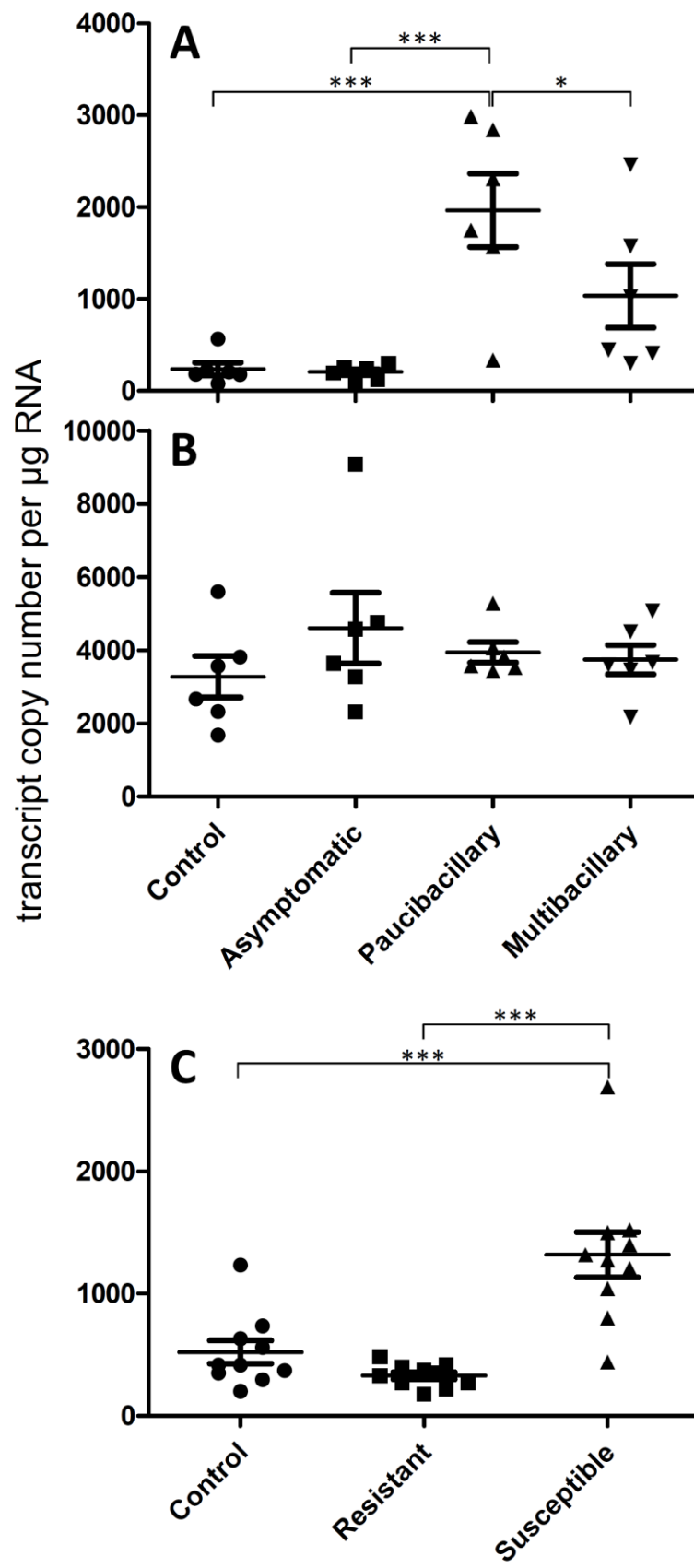


Table S1

Pathology, breed and age of sheep

Pathology	Breed	Age (y)
Pauci	Blackface × Bleu du Maine	4.5
Pauci	Texel	5.5
Pauci	Blackface × Bleu du Maine × Lleyrn × Roussin	2.5
Pauci	Lleyrn x Roussin	3.5
Pauci	Texel	2.5
Pauci	Blackface	2.5
Multi	Blackface	3
Multi	Blackface	4
Multi	Blackface	4
Multi	Blackface	3
Multi	Blackface	3
Multi	Blackface	5
Asympto	Blackface × Bleu du Maine	7
Asympto	Blackface × Bleu du Maine	7
Asympto	Greyface	4
Asympto	Greyface	4
Asympto	Greyface	4
Asympto	Greyface	4
Controls	Blackface	4.5
Controls	Blackface	4.5
Controls	Blackface	4.5
Controls	Blackface	4.5
Controls	Blackface	4
Controls	Texel	4